APPLICATION

FOR

UNITED STATES LETTERS PATENT

BY

ELAZER R. EDELMAN

ARUNA NATHAN

AND

MATTHEW A. NUGENT

FOR

INHIBITION OF VASCULAR OCCLUSION FOLLOWING VASCULAR INTERVENTION

INHIBITION OF VASCULAR ~~OCCLUSION~~ ~~FOLLOWING INTERVENTION~~

Background of the Invention

The United States government has rights in this invention by virtue of National Institutes of Health grants GM/HL 49039 and AG00294, to Elazer R. Edelman.

The present invention is generally in the area of methods for inhibiting or preventing vascular smooth muscle cell proliferation, or restenosis, following vascular intervention or injury, such as angioplasty, vascular bypass surgery, organ transplantation, or other vascular intervention or manipulation.

Angioplasty, surgery and other vascular intervention are complicated by an accelerated arteriopathy characterized by rapid growth of cells into the lumen within a short period of time which is severe enough to jeopardize the blood flow to distal organs.

Vascular bypass surgery has been widely used to correct stenotic and occluded blood vessels, as when plaques develop on the surface of blood vessels in atherosclerosis. In bypass surgery, one or more healthy blood vessels are grafted into the occluded vessels at either end of the occlusion to shunt blood around the stenotic or occluded vessel to re-establish a sufficient blood supply to the tissue whose blood supply is endangered by the stenosis or occlusion. This surgery often successfully revascularizes the endangered tissue.

In recent years, angioplasty has been developed as an alternative treatment to bypass surgery, especially in patients who have been diagnosed early in the development of stenosis or occlusion of blood vessels due to the abnormal laying down of plaque on the endothelial wall of a blood vessel. Angioplasty

typically involves guiding a catheter which is usually fitted with a balloon or expandable metal mesh up through an artery to the region of stenosis or occlusion and the brief inflation, one or more times, of the balloon or wire mesh to push the obstructing intravascular material or plaque up against the endothelial wall of the vessel, thereby compressing and/or breaking apart the plaque and reestablishing blood flow. However, angioplasty treatment can injure the vessel, especially when the balloon is overinflated or the mesh overextended, causing a variety of undesirable results, such as denudation (removal) of the endothelial cell layer in the region of the angioplasty, dissection of part of the inner vessel wall from the remainder of the vessel with accompanying occlusion of the vessel, or rupture of the *tunica intima* layer of the vessel.

Though the hallmark of the rapidly accelerating lesions following angioplasty, vascular bypass grafting, and organ transplantation is the proliferation of smooth muscle cells and their accumulation within the *tunica intima*, it is the loss of normal endothelial function that heralds these events and may stimulate them to occur. The arterial endothelium serves as a transport barrier, a biochemical filter and as a regulator of many vascular phenomena. The most potent vasodilators, thromboresistant compounds and inhibitors of smooth muscle cell proliferation, are endothelial derived. Vascular smooth muscle cell accumulation within the intima ceases with restoration of the endothelium (Schwartz et al., *Am. J. Pathol.*, *81*: 15-42 (1975); Fishman et al., *Lab. Invest.*, *32*: 339-51 (1975)) and regression of intimal hyperplasia is maximized where

3

endothelial restoration is maximized (Bjornsson et al., *Proc. Natl. Acad. Sci. USA*, *88*: 8651-8655 (1991)). Confluent, and not exponentially growing, endothelial cells produce a series of compounds that are the most potent vasodilators, inhibitors of spasm, and inhibitors of smooth muscle cell proliferation. Heparan sulfate proteoglycan produced by the endothelial cells has multitudinous effects on the smooth muscle cells including interfering with binding of heparin-binding growth factors (Nugent et al., *Circulation Research*, *73*: 1051-1060 (1993), which are known to stimulate vascular smooth muscle cell growth (Nugent et al., *Circulation Research*, *73*: 1051-1060 (1993); Castellot et al., *J. Cell Biol.*, *90*: 372-9 (1981)). It appears, therefore, that restoring the endothelial monolayer of a blood vessel restores the agents or compounds responsible for biochemical control of vascular cell proliferation.

Other efforts at limiting the undesirable proliferative and disease states of vascular endothelium have focused on the isolated administration of analogs of endothelial compounds. Certain drugs, such as heparin, are especially effective inhibitors of vascular smooth muscle cell proliferation in tissue culture and animal models of arterial diseases precisely because they mimic the activity of natural endothelial-derived compounds like heparan sulfate proteoglycan, Edelman, E.R. & Karnovskv, M.J. *Circ.* 89: 770-776 (1994). However, despite cell culture and small animal data supporting the regulatory role of heparin-like compounds, exogenous heparin preparations have shown no benefit in human trials. For example, when patients were randomized to heparin or dextrose infusion over the

first 18 to 24 hours post angioplasty, 41.2% of the heparinized patients and only 36.7% of the dextrose infusion patients had evidence for restenosis (Ellis et al., *Am. Heart. J., 117*: 777-782 (1989)). Moreover, bleeding complications were twice as frequent in the heparinized group. In another trial, angioplasty patients injected subcutaneously with heparin at 10,000 IU/day had 2.5 fold more restenosis and significantly more ischemic complications than patients treated in the standard fashion (Lehmann et al., *J. Am. Coll. Cardiol., 17*: 181A (abstract) (1991)). Non-heparin endothelial compounds such as nitric oxide and the prostaglandins are potent regulators of a range of biologic effects involving smooth muscle cells. Inhibitors of these compounds have been shown to control intimal hyperplasia following experimental vascular injury (Cooke et al., *Curr. Opin. Cardiol.*, 7: 799-804 (1992); Moncada et al., *N. Engl. J. Med., 329*: 2002-2012 (1993); McNamara, et al., *Biochem. Biophys. Res. Comm., 193*: 291-296 (1993)). This is indicative that the vascular endothelium is a powerful regulator of the blood vessel wall, not because of the production and secretion of one compound alone, but because of its presence as an intact unit. While concerns have been raised as to the difference in protocols allowing for benefit in animals and worsening of disease in man (Edelman et al., *Circulation, 89*: 770-776 (1994)), it appears likely that a single endothelial product, such as heparin, is incapable of replacing the entire endothelium in the control of complex human vascular lesions.

Accordingly, there is a need for means and methods of promoting healing of vascular tissue and

controlling vascular muscle cell proliferation (hyperplasia) to prevent restenosis of blood vessels after angioplasty, vascular bypass, organ transplantation, or vascular disease without the risk of rapid reocclusion.

It is therefore an object of the present invention to provide a means of preventing occlusion following vascular intervention.

It is a further object of the present invention to provide methods for understanding and controlling the mechanisms of restenosis and vascular occlusion.

Summary of the Invention

Compositions and methods for inhibiting excessive intimal growth (hyperplasia) and/or vascular smooth muscle cell proliferation (hyperplasia) following intravascular intervention or injury are disclosed. In the preferred embodiment, endothelial cells are present in or on a biocompatible, biodegradable or non-biodegradable matrix, which allows the cells to reside and secrete products into the surrounding tissue or into the matrix, from which they can diffuse into the surrounding tissue. The endothelial cell-matrix composition is juxtaposed with the target blood vessel, which might be injured, for example, from angioplasty, surgery, transplantation or atherosclerosis, to inhibit subsequent restenosis or occlusion of the blood vessel.

Brief Description of the Drawings

Figure 1 is a graph of the growth (measured as cell number) of bovine aortic endothelial (BAE) cells

(open circles) and Chinese Hamster Ovary 745 cells (controls which do not produce regulators of smooth muscle cell proliferation) (dark circles) on Gelfoam™ (The Upjohn Co., Kalamazoo, MI) collagen matrix over time (days).

Figure 2 is a graph of inhibition (% control) of binding (dark circles) and mitogenesis (dark squares) of the potent vascular cell growth promoter bFGF for vascular smooth muscle cells by endothelial cell-Gelfoam® conditioned media, compared with inhibition of bFGF binding (open circles) and mitogenesis (hatched squares) for vascular smooth muscle cells by CHO-745 cell-Gelfoam® conditioned media.

Figure 3 is a graph of the ratio of the area of the *tunica intima* to the area of the *tunica media* after balloon denudation of the endothelium (BI) when the balloon injured arteries were exposed to Gelfoam® alone (Gel), Gelfoam® engrafted with CHO-745 cells (CHO), Gelfoam® containing heparin (Hep), and Gelfoam® containing endothelial cells (EC).

Figure 4 is a graph showing cell proliferation within both the *tunicae intima* and *media*, when exposed to either Gelfoam® engrafted with endothelial cells (EC), Gelfoam® with heparin (Hep), Gelfoam® engrafted with CHO-745 cells (CHO), Gelfoam® control or untreated following balloon angioplasty (BI), comparing the % proliferating cells for the *tunica intima* (open bar) and *tunica media* (dark bar).

Detailed Description of the Invention

The compositions described herein for inhibition of intimal hyperplasia consist of a matrix, most

preferably a hydrogel, seeded with endothelial cells which can be xenografts, allografts or autografts, which is implanted at a site near to or at the region of hyperplasia at the time of vascular intervention or upon diagnosis of restenosis or other occlusion of a blood vessel.

Compositions to Inhibit Intimal Hyperplasia

The compositions described herein are used to inhibit undesired response to vascular injury that includes hyperplasia of smooth muscle vascular cells which occurs in response to injury to the endothelial tissue of blood vessels, for example, as a result of angioplasty, coronary artery bypass used to open a stenotic or occluded vessel. Vascular smooth muscle cell hyperplasia triggered by the injured endothelium can result in restenosis of the blood vessel due to the excessive proliferation of vascular smooth muscle cells.

Cells

Endothelial cells are isolated by standard methods, for example, as described by Gimbrone, M. Culture of vascular endothelium. *Progress Hemostasis and Thrombosis* 3:1-28 (1976). Cells can be obtained at the time of the procedure using standard biopsy techniques, whether the procedure is angioplasty, open field surgery or for diagnostic purposes. The cells can be dissociated with collagenase or trypsin and seeded directly into a matrix as described below for immediate implantation, or cultured *in vitro* as required to generate the number of cells to be implanted. The cells are typically seeded into or onto the matrix at a density of between approximately 10^3 and 10^{12} cells/cm^3. Cell densities can be determined using visual methods or a Coulter counter.

The effective amount of cells and matrix to be administered is that which prevents or inhibits hyperplasia at the site of vascular injury.

Other cells which are genetically engineered, using oligonucleotides resulting in ribozyme-mediated cleavage of targeted genes or regulatory elements, or antisense to block transcription of targeted genes, or sequences encoding defective, missing or insufficient genes, can also be used. For example, cells transfected with genes for synthesis of heparan sulfate, regulators of heparan sulfate, nitric oxide synthase, growth factors, cytokines and other vasoregulatory products could be seeded into the matrix and implanted. Genes can be transfected into the cells using standard methodology such as viral vectors, microinjection and calcium phosphate precipitation.

Other materials can also be administered via the matrix, including anti-inflammatory agents, prostaglandins, prostanoids, angiotensin and related compounds, tyrosine kinase inhibitors, immunosuppressants, vitamins, glucocorticoids, anti-oxidants, free radical scavengers, peptide hormones, angiogenic and angiogenic inhibitory factors.

The Matrix

The endothelial cells are seeded onto or into a biocompatible matrix which is suitable for implanting in contact adjacent to or at the site of the vascular injury, for example, by wrapping around the blood vessel. The matrix can be in the form of a gel, foam, suspension, microcapsules, solid polymeric support, or fibrous structure. The matrix may also serve in a physically supporting role. There is no specific requirement as to thickness, size or shape. When the

cells are seeded within the matrix, it is preferred that the matrix be sufficiently porous to allow free diffusion of nutrients and gases into the matrix to maintain cell viabilility, while allowing the secretory products of the cells to diffuse out of the matrix into the surrounding tissue in roughly physiologically quantities. The matrix may also serve to protect non-autologous cells from immune attack.

Preferably, the matrix is a biodegradable material, such as a synthetic polymer degrading by hydrolysis, for example, polyhydroxy acids like polylactic acid, polyglycolic acid and copolymers thereof, polyorthoesters, polyanhydrides, proteins such as gelatin and collagen, or carbohydrates or polysaccharides such as cellulose and derivatized celluloses, chitosan, alginate, or combinations thereof, so that over the course of several days or weeks after implantation of the matrix material, the matrix gradually disappears. In a preferred embodiment, the matrix is a hydrogel, defined as a matrix wherein typically approximately 90% by weight of the matrix is absorbed water. Hydrogels can be formed by ionic or covalent crosslinking of a variety of water soluble polymers such as polyphosphazenes, polysaccharides such as alginate, and proteins such as gelatin. As demonstrated in the following examples, a presently preferred matrix material is purified gelatin-based Gelfoam™ (The Upjohn Co., Kalamazoo, MI) surgical sponge.

The use of biodegradable matrices eliminates the need for surgery to remove undegraded implanted matrix, after regression of the hyperplasia and healing of the endothelial injury. However, synthetic non-biodegradable matrices may also be used. Useful

10

materials include ethylene vinyl acetate, polyvinyl alcohol, silicone, polyurethane, non-biodegradable polyesters, and polyethyleneoxide-polypropyleneoxide, and tetrafluoroethylene meshes (Teflon®).

Attachment of the cells to the matrix can be enhanced by coating or chemically modifying, attaching or derivatizing the polymer with a material such as collagen, laminin, fibronectin, fibrin, basement membrane components, and attachment peptides (RGD and many others are known and described in the literature).

Culturing of Cells in Matrix

The matrix material seeded with endothelial cells can be cultured *in vitro* to promote cell growth throughout the matrix. At various times during incubation of the seeded matrix in the culture medium, samples of seeded matrix can be removed and examined, for example, by cell staining and microscopic observation, to determine the extent of cell growth and proliferation in the matrix, as well as production of total sulfated glycosaminoglycans and, specifically, heparan sulfate.

When the cells have reached the desired cell density in the matrix or have confluently grown over most of the interstices of the cultured matrix, the matrix is removed from the culture medium and either used in the procedures to treat vascular smooth muscle cell hyperplasia described below, or stored for subsequent implantation.

Alternatively, the matrix is seeded with the cells immediately prior to implantation of the matrix.

Methods of Treating Intimal Hyperplasia

Patients can be diagnosed for vascular endothelial cell injury using known methods, such as X-ray fluoroscopic examination of dye flowing through a particular region of a blood vessel or other visual techniques, the presence of symptoms such as pain, based on clinical judgment, or signs evidenced physical examination. Alternatively, it can be assumed that injury will arise due to performance of procedures such as angioplasty, arterial bypass graft, peripheral bypass surgery, or organ transplantation and the patient treated based on the assumption that injury or disease will inevitably arise.

In a preferred embodiment, endothelial cell-matrix strips are applied to the site of injury during open field surgery. In this embodiment, the strips of cultured matrix are applied to the exterior of the injured blood vessel over the internal site of injury, usually by wrapping the matrix strips around the vessel. After implantation, the cells remain viable and produce factors such as sulfated glycosaminoglycan, including heparan sulfate.

If intimal hyperplasia had been observed prior to implanting or wrapping the strips of matrices, the regression of hyperplasia is typically evidenced by a decrease in pain or other symptoms of decreased blood flow, or through the use of imaging techniques. The decrease in hyperplasia or increase in flow rate through the injured vessel can be monitored by the same methods used to initially diagnose the injury to the vascular endothelium or blockage of the blood vessel.

The following non-limiting examples illustrate some of the various aspects of compositions and

methods used to treat vascular smooth muscle cell hyperplasia and restenosis of blood vessels.

Example 1: Cell Engraftment and Characterization of Cells Cultured in the Seeded Matrix.

Bovine aortic endothelial (BAE) and mutant Chinese hamster ovary (CHO-745) cells were cultured on Gelfoam™ matrices. The mutant cells, obtained from Dr. J. Esko (University of Alabama, Birmingham, AB), served as control cells as they produce no demonstrable heparan sulfate (Esko 1992) and, as a result, unlike endothelial cells, do not inhibit bFGF binding to or mitogenesis of cultured vascular smooth muscle cells (Nugent, et al., 1993). Gelfoam™ has long been used as an implantable surgical sponge and more recently as a scaffolding for cell growth (Centra, et al., 1992). This material, isolated from porcine dermal gelatin, was supplied in blocks (UpJohn Inc.), cut into 2.5 x 1 x 0.3 cm^3 pieces and hydrated by autoclaving for 10 min in Hanks balanced salt solution (HBSS). Upon cooling, the blocks were placed in 17 x 100 mm polypropylene tubes to which 2 mL of cell suspension (0.6 x 10^5 cells/mL) was added. The endothelial cells were suspended in Dulbecco's modified Eagle's medium (DMEM) and the CHO-745 cells were suspended in Ham's F-12 medium. Media was supplemented with 1 g/L glucose and 10% calf serum.

Culture tubes were gently agitated to disperse the cells and then incubated at a 45° angle, 37°C, humidified, 5% CO_2 and 95% air, for up to 15 days. Growth medium was changed on days 3, 7 and 12. On days 0, 2, 4, 7, 10 and 15, the number of cells in the media (non-adherent cells) and those attached to the Gelfoam™ were determined using a hemocytometer. Prior to counting the attached cells, the Gelfoam™

blocks were washed four times with HBSS to remove non-attached cells and serum. Collagenase (1 mg/mL) was used to digest the Gelfoam™, releasing all the cells from the matrix. An 80 μL aliquot was removed for cell counting and the viability was checked by trypan blue exclusion.

The results are shown in Figure 1. Each data point represents the average cell number ± the standard error (SEM) of duplicate determinations.

Cells lined the interstices of this three dimensional collagen-like matrix (Centra, et al., 1992), and remained within the matrices without migrating outward. Cell growth followed a pattern similar to that observed on tissue culture polystyrene. Cell viability as evaluated by trypan blue exclusion remained at 90 ± 2.3% for the BAE cells and 93.1 ± 1.7% for the CHO-745 over the course of the culture.

The preservation of the immune identity of cells within Gelfoam™ blocks recovered 14 days after placement in tissue culture or around rat carotid arteries was determined by immunostaining for the endothelial marker von Willebrand's factor as described by Edelman, et al., *J. Clin. Invest.* 89:465-471 (1992).

The amount of heparan sulfate in conditioned media produced by cells cultured on Gelfoam™ or tissue culture polystyrene was measured. Gelfoam™ films containing endothelial cells or CHO-745 cells were incubated in culture medium containing no antibiotics and no calf serum for 24 hours at 37°C. As a control, identical Gelfoam™ films without cells were incubated in the respective media. The medium was collected, centrifuged (5,000 x g), dialyzed

exhaustively against water, and concentrated by lyophilization. Total sulfated glycosaminoglycan was determined using dimethylmethylene blue (Farndale, et al., *Biochimica et Biophysica Acta* 883:173-177 (1986), and the amount of heparan sulfate was assessed after samples were treated with heparinase. The endothelial and CHO-745 cells grown on Gelfoam™ were also radiolabeled with $^{35}SO_4$ (100 μCi/mL, 24 h) to visualize the metabolic synthesis of heparan sulfate by the cells. The medium was collected, centrifuged, and $^{35}SO_4$-proteoglycan separated from the free $^{35}SO_4$ by vacuum filtration through cationic nylon membranes (Rapraeger and Yeaman, *Anal. Biochem.* 179:361-365 (1989)). Filters containing $^{35}SO_4$-proteoglycan were incubated in nitrous acid (0.48 M sodium nitrite combined with 3.6 M acetic acid) and counted to determine the amount of radioactivity incorporated within heparan sulfate.

Engrafted cells remained viable with full retention of biochemical secretory ability and biologic potency as well as immunoidentity. Cells cultured on Gelfoam™ produced nearly identical amounts of total sulfated glycosaminoglycan and heparan sulfate as that produced when the same cells were grown on polystyrene dishes. In addition, there was no significant difference in the profile of the proteoglycan when resolved on 5% SDS-PAGE. For both cells grown on Gelfoam™ and tissue culture polystyrene, the majority of the proteoglycan migrated as a band greater than 600 kDa relative to protein standards. When compared to CHO-745 cells grown on Gelfoam™, the engrafted endothelial cells produced an 11.1-fold greater amount of glycosaminoglycan (4.1 $\pm$ 0.3 microgram/10^6 cells/day for endothelial cells

versus 0.37 ± 0.02 microgram/10^6 cells/day for CHO-745 cells). While 29.1% of the glycosaminoglycan produced by the endothelial cells was heparan sulfate (1.2 ± 0.05 microgram/10^6 cells/day), CHO-745 cells produced no detectable heparan sulfate. Conditioned media from Gelfoam™ engrafted endothelial cells also inhibited both binding of ^{125}I-bFGF to heparan sulfate proteoglycan and the mitogenic effect of bFGF on vascular smooth muscle cells in a dose dependent fashion, as shown by Figure 2. In contrast, conditioned media from CHO-745 cells had no effect on binding, and similarly no effect on growth factor induced mitogenesis, as shown by Figure 2.

Example 2: Inhibition of Intimal Hyperplasia in Rats.

Implantation of Matrix Strips Containing BAE Cells into Rats.

The *in vivo* effects of engrafted cells in matrix strips were evaluated in an endothelial denuding arterial injury model in Sprague-Dawley rats. Strips of Gelfoam™ gelatin matrix (2.5 x 1 x 0.3 cm^3 each) containing BAE cells or CHO-745 cells, or no cells, were wrapped around carotid arteries denuded with a balloon catheter, and their effect on smooth muscle cell proliferation and intimal hyperplasia was determined two weeks later.

Endothelial denudation of the left common carotid artery in Sprague-Dawley rats was performed with a 2 French Fogarty balloon catheter introduced through an external carotid arteriotomy and passed three times in its inflated state over the endothelium of the common carotid artery (Edelman, et al., 1992, Clowes, et al., 1983, Edelman, et al., 1990). Strips of Gelfoam™

(2.5 x 1 x 0.3 cm^3 each) containing BAE cells, CHO-745 cells, or no cells were wrapped around denuded arteries. The strip ends overlapped, ensuring complete encircling of the artery. Fascial planes were sutured closed to further immobilize the devices.

To compare the results of anticipated physiologic secretion of endothelial cell products to the pharmacologic dosing of a proven endothelial-cell analog, hydrogel films were formulated (Nathan, et al., 1994) to release heparin alone at a dose, 2.5 $\pm$ 0.1 microgram/day. This dose and mode of delivery has previously been demonstrated to maximize heparin's inhibition of neointimal hyperplasia (Edelman, et al., 1990; Edelman, et al., 1993; Edelman and Karnovsky 1994).

On the 14th post-operative day, animals were euthanized and perfused clear via the left ventricle with Ringer's lactate solution followed by immersion fixation with Carnoy's fixative (60% methanol, 30% chloroform, 10% glacial acetic acid). The location of the implanted matrices was noted and the matrices recovered with the entire length of the intact arteries. The carotid arteries were harvested and cut into five equal segments, three including the Gelfoam™ wrap and segments above and below. Segments were paraffin embedded and 6 micron sections obtained along the length of each segment.

After staining with hematoxylin/eosin or verHoeff's elastin stain, the intimal, medial and adventitial areas, the intima:media area ratio and the percent of luminal occlusion were calculated using computerized digital planimetry with a dedicated video microscope and customized software. Cell proliferation was assayed using immunocytochemical

identification of proliferating cell nuclear antigenicity (PCNA) and the thymidine analog 5-bromo-2'-deoxyuridine (BrdU, New England Nuclear, Dupont Corp., Wilmington, DE) which had been injected intraperitoneally, at 50 mg/kg body weight, 3 and 7 days post-surgery and one hour prior to sacrifice.

Segments of the unwrapped balloon injured artery were compared to Gelfoam™ wrapped segments. Comparisons were made across treatment groups and within treatment groups to allow each animal to serve as its own control. Statistical comparisons were performed using analysis of variance (ANOVA) and subsequent differences among groups using Student's t test. Data was rejected as not significantly different if p values of greater than 0.05 were observed. Data line fits were established using a linear regression and correlation model.

The *in vivo* potency of the endothelial cell engrafts was retained in addition to their *in vitro* effects. Balloon denudation of the carotid arterial endothelium led to an increase in the ratio of the area of the *tunica intima* to the area of the *tunica media* (I:M) to 1.44 $\pm$ 0.16, as shown by Figure 3. Arterial wall segments exposed to Gelfoam™ containing endothelial cells showed minimal hyperplastic disease. Intimal hyperplasia in arterial segments beneath endothelial cell grafted Gelfoam™ was reduced to 0.17 $\pm$ 0.07. This modulating effect was specific to endothelial cells. Control CHO-745 cell engrafts had no statistically significant effect on intimal hyperplasia (I:M 1.36 $\pm$ 0.32) compared to balloon injury, alone or empty Gelfoam™ matrices (I:M 1.20 $\pm$ 0.11), as shown by Figure 3.

Heparin has been identified as the gold standard inhibitor of smooth muscle cell proliferation and intimal hyperplasia, in major part because it resembles endothelial-cell-derived heparan sulfate proteoglycan. Indeed the perivascular release of heparin alone from hydrogel films reduced proliferation to an I:M of 0.55 $\pm$ 0.11. This result, however, was 3.2 fold less effective than the control exerted by engrafted endothelial cells despite hydrogel release of heparin at twice the rate of release of heparan sulfate proteoglycan from the endothelial implants.

Gelfoam™ engrafted endothelial cells reduced proliferation as well as intimal hyperplasia. The number of proliferating cells relative to the total number of cells in the *tunicae media* and *intima* were used as an index of proliferation. Gelfoam™ implants with engrafted endothelial cells caused a statistically significant decrease in cell proliferation in both the *tunica intima* (20.4% reduction) and the *tunica media* (26.3% reduction) relative to empty Gelfoam™ implants, as shown by Figure 4. Heparin infusion also decreased proliferation but the effect was not statistically significant at the number of animals and sections examined. Relative to balloon injury alone, heparin infusion reduced BrdU immunostaining in the *tunica intima* by 11.0% and 12.2% in the *tunica media*, as shown by Figure 4.

There was no evidence that the implants induced cellular or systemic rejection. The general health and appearance of the animals with the implants was unchanged from controls. Rats subjected to balloon injury alone gained 52.9 $\pm$ 3.6 gms over the 14 day

experimental period, and those animals that received a cell-free implant hydrated in either DMEM or HAMS-F12 gained 48.0 ± 7.3 gms. The animals implanted with endothelial-seeded implants gained 51.3 ± 2.8 gms and the animals with CHO-745 laden implants gained 53.4 ± 6.5 gms. Rats receiving perivascular heparin gained 56.7 ± 6.2 gms, indicating that the treatment had no side effects.

The results demonstrate the engraftment of endothelial cells on three dimensional biopolymer scaffoldings with preservation of cell viability, normal growth characteristics, immunologic markers, biochemical activity and physiologic effects.

The biologic effects of the engrafted endothelial cells were cell-specific and superior to the infusion of a single pharmacologic analog of an endothelial product, heparin. Conditioned media from implant engrafted endothelial cells, but not heparan sulfate proteoglycan-deficient CHO-745 cells, inhibited bFGF binding to and mitogenesis for vascular smooth muscle cells in a dose dependent fashion. Similarly, only endothelial cell engrafts inhibited intimal hyperplasia. The implant alone or seeded with CHO-745 cells had no statistically significant effect on cell proliferation or intimal hyperplasia. Heparin is one of the most effective anti-proliferative agents for vascular smooth muscle cells, yet even when released at twice the rate of heparan sulfate proteoglycan production by the endothelial cells, intimal hyperplasia and smooth muscle cell proliferation were reduced 3.2 fold less effectively than with the endothelial cell implants.

The control exerted by the endothelial cell implants appears to result solely from the biochemical

effects of the engrafted cells. Immunostaining with endothelial cell-specific markers detected no evidence for early recovery of endogenous endothelial cells or the migration of engrafted cells from their biopolymer scaffoldings to the arterial lining in any of the recovered arterial segments. The effects of the implants were localized in a similar manner to the focal effects observed with the perivascular release of other compounds. There was no evidence of a systemic or local immune response or graft rejection.

Modifications and variations of the present invention will be apparent to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.